Preliminary pharmacokinetic study of the anticancer 6BIO in mice using an UHPLC-MS/MS approach

Short title: Pharmacokinetic study of 6BIO in mice using UHPLC-MS/MS

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Abstract

Indirubins represent a group of natural and synthetic products with bio-activities against numerous human cancer cell lines acting by inhibiting protein kinases. The natural sources of indirubins are plants of *Isatis sp.*, *Indigofera sp.*, and *Polygonum sp.*, recombinant bacteria, mammalian urine and some marine mollusks. Specifically, the halogenated derivative 6-bromo indirubin-3′-oxime (6BIO) possesses increased selectivity against GSK-3. However, to our knowledge, no analytical method to determine 6BIO in biological fluids has been developed till now. Therefore, a rapid, sensitive and high throughput UHPLC-MS/MS methods were developed and validated to evaluate the concentrations of 6BIO in mice plasma. Plasma samples were pre-treated by protein precipation using cold mixture of methanol: acetonitrile (9:1, v/v) and separations were carried out on a Hypersil Gold C18 column (50 × 2.1 mm i.d.; 1.9 µm p.s.) using 0.1% acetic acid and methanol as mobile phase at a flow rate of 500 mL/min in a gradient mode. For quantitation, a hybrid LTQ-Orbitrap MS equipped with an electro-spray ionization source was used applying a selected reaction monitoring (SRM) option. The monitored transitions were m/z 354.0 → 324.0 for 6BIO and 297.1 → 282.1 for afromorsin (used as the internal standard) in the negative mode. Following the EMA, ICH and FDA guidelines for validation of analytical procedures, the assay method was fully validated in terms of selectivity, linearity, recovery, matrix effect, accuracy, precision, stability, and robustness. The validated methods were successfully applied to the pharmacokinetic studies of 6BIO following an oral administration to mice at the dose of 50 mg/kg. The results indicated that 6BIO possesses a T_{max} of 30 min, a half-life of 1 h, and low plasma bioavailability.

Keywords: 6BIO, pharmacokinetics, UHPLC-Orbitrap MS, quantitation, plasma

1. Introduction

Indirubins are bis-indole alkaloid compounds occurring naturally in various indigo-containing plants such as *Isatis spp* – Brassicaceae (1, 2), *Indigofera spp* – Leguminoseae (3), *Polygonum spp* – Polygonaceae (4), *Strobilanthes spp* - (5). Besides their importance in the dye industry history, these compounds display important biological activities with a particular focus on the inhibition of protein kinases (6). A secondary natural source of indirubins are mollusk gastropods from Muricidae family such as *Hexaplex trunculus* (7), *Plicopurpura spp* (8), and others (9). Plant indirubin have been found as the active principle of the anti-leukemial Traditionnal Chinese Medicine (TCM) *Danggui Longgui Wan* (10-11), with a particular action against Cyclin-Dependent Kinases (12). On the other hand, the mollusk derived halogenated analogue, 6-bromo indirubin (6BI) inhibits preferably the mamalian Glycogen Synthase Kinase-3 (GSK-3) (13, 14). The anti-protein kinase activities of natural occurring indirubins have attracted the attention of the scientific community this last decade, leading to a particular increase of interest in the study of the biology and the chemistry of indirubins as reviewed in (15-17). Therefore, hundreds of indirubin derivatives have been developed during the recent years, aiming towards the investigation and amelioration of the activity, selectivity and drug-likeness of indirubins (18-22).

6-bromoindirubin-3′-oxime (6BIO) is a semi-synthetic analogue of 6BI that was developed as a potent and selective GSK-3β inhibitor (23) and is currently considered as a prototype inhibitor of that mammalian kinase. 6BIO has also been used to maintain pluripotency of mouse and human embryonic stem cells through the reversible Wnt pathway activation, suggesting therefore its applications in regenerative medicine (24). Besides GSK3β inhibition, 6BIO also inhibits JAK/STAT3 signaling, induces apoptosis of melanoma cells *in vitro* and suppresses tumor growth

in vivo with low toxicity in a mouse xenograft model of melanoma (25). This anti-cancer like activity of 6BIO has been also investigated in the metastatic process of cancer cell lines *in vitro*: 6BIO inhibited adhesion, migration and invasion of a variety of metastatic cell types by inhibiting several kinase cascades such as PDK1, GSK3β and Jak/STAT3 (26). These results were confirmed by an *in vivo* experiment where 6BIO decrease significantly lung metastasis in the well-established 4T1 mouse model of aggressive breast cancer after a treatment at the dose of 1 mg/kg. Another reported mode of action of 6BIO is its potential to fight TRAIL (TNFα-related apoptosis-inducing factor) resistant cancer cells (27). Futhermore, 6BIO was recently found to be a full agonist of AhR, it participates, through AhR signaling, to the induction of Cytochrome P450-1A2 which is involved in one of the key metabolic functions of the liver (Briolotti et al., 2015). Moreover, the commercialization of 6BIO by Calbiochem (Merck-Millipore) and Santa Cruz Biotechnology under the name GSK-3 Inhibitor IX (CAS 667463-62-9) is a clear indication of its value as a tool for the exploration of physiological pathways. Despite the pleiotropic effects of indirubin derivatives and 6BIO in particular, scientific reports regarding the determination of 6BIO in biological fluids are very scarce.

To further explore the potential of 6BIO, a bioanalytical method was needed in view of pharmacokinetic studies with this compound. We therefore report here the development and validation of an UHPLC-ESI-HRMS/MS method for the determination of 6BIO in mice plasma. The use of UHPLC ensured the short analysis time (4.4 min), whereas the hybrid LTQ-Orbitrap Mass Spectrometer and the application of the SRM option offered high mass measurement accuracy and exclusion of any possible matrix interference and false positives from endogenous components having the same nominal mass. Overall, the developed methodology is suitable for high-throughput analysis requirements in the context of simple and efficient extraction with small

plasma volume, reduced analysis time and high sensitivity, all being necessary requirements for accessing the pharmacokinetic profile of the drug. The developed methodology has been used to access the pharmacokinetic profile of 6BIO after its administration in healthy mice.

2. Material and methods

2.1 Chemicals and reagents

6BIO (Figure 1) was synthesized as previously described (35). Afrormosin (I.S.) was isolated as previously described (28). Glacial acetic acid and LC-MS grade water were purchased from Merk (Damstart, Germany). LC-MS grade acetonitrile and methanol were purchased from Carlo Erba (Val de Reuil, France).

2.2 Ultra-high performance liquid chromatography

The samples were analyzed on a UHPLC-LTQ-Orbitrap Discovery system (Thermo Scientific, Bremen, Germany) consisted of an Accela UHPLC system coupled to a hybrid LTQ-Orbitrap Discovery Mass spectrometer. An aliquot of 10 µL of sample was injected onto a C_{18} Hypersil GOLD column (50 × 2.1 mm i.d., 1.9 µm particle size, Thermo Scientific) with a flow rate of 500 µL/min at room temperature. The LC linear gradient was increased from 10% B to 55% B in 2 min (A, water with 0.1% acetic acid; B, acetonitrile), held at 55% B for 0.3 min, and then increased to 100% B in 1 min, restored to 10% in 0.1 min and held at 10% B for 1 min. The total run time was 4.4 min.

2.3 Mass spectrometry

Mass spectrometric detection was carried out on an LTQ-Orbitrap Discovery Mass Spectrometer (Thermo Finnigan) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 354.0 → 324.0 and *m/z* 297.1 → 282.1 for 6BIO and Afrormosin respectively (Figure 2). The peak area ratio of 6BIO to the IS was used for the quantification of 6BIO in plasma samples. The optimized ionization parameters were as follows: ion spray voltage of 3.5 kV, temperature 300 ^{o}C, capillary voltage -50 V and tube lens -50 V. Two microscans were applied with a maximum injection time of 100 ms. Nitrogen was used as sheath gas (flow rate 50 arbitrary units) and auxiliary gas (flow rate of 10 arbitrary units). All samples were analysed in duplicate and data were acquired and processed using the Xcalibur 2.0 software.

2.4 Standard solutions, calibration standards and quality control samples

A stock solution of 6BIO was prepared by dissolving 5 mg of crystaline 6BIO in 5 ml of DMSO. The stock solution of the IS (Afrormosin) was prepared by dissolving 5 mg of the IS in 5 mL of methanol. Acetonitrile and water were used as solvent in the ratio of 1:1 (v/v) throughout the analysis. The stock solution of 6BIO was further diluted to give a series of sub-standard solutions with the concentrations of 50, 100, 250, 500, 1000, 1250, 1500 and 2000 ng/mL. The IS stock solution (1.0 mg/mL) was further diluted in methanol (HPLC grade) to yield a working standard solution of 500 ng/mL and subsequently stored at -20 ^{o}C pending analysis. The calibration standards of 6BIO were prepared by spiking 0.45 µL of 9 series of analyte-free mice plasma with 5 µL of the 9 series of appropriate 6BIO working standard solutions leading to plasma with a new

series of concentrations (5.0, 10, 25, 50, 100, 125, 150 and 200 ng/mL). LLOQ (5 ng/mL) and the ULOQ (200 ng/mL) were prepared in pentaplicate. Similarly, the quality control (QC) samples were prepared in pentaplicate using analyte-free mice plasma at concentrations of 7.5, 40 and 175 ng/mL.

2.5 Sample preparation

Protein precipitation was used for the treatment of plasma samples prior to the LC-MS analysis. 25 μL of the IS working solution was added to 225 μL of acetonitrile, vortexed for 30 s and maintained at -20 ºC. This new IS solution of 50 ng/mL (acetonitrile/methanol: 9/1, v/v) was added to 50 μL of the plasma samples containing the analytes. The resulting suspension vortex-mixed for 1 min, then centrifuged for 5 min (12000 rpm) and finally 10 μL of the supernatant were injected into the UHPLC-LTQ-orbitrap system.

2.6 Method validation

The method was validated for selectivity, carry-over, linearity, accuracy, precision, dilution integrity, robustness, matrix effect, extraction recovery and stability according to the International Conference on Harmonization (ICH, 2005) Q2 (R1) procedures, the Bioanalytical Method Validation Draft Guidance of the US Food and Drug Administration (FDA, 2013) and the Guideline Bioanalytical method validation of the European Medicines Agency (EMA, 2011). The peak area ratios of 6BIO to the I.S. of QC samples were interpolated from the calibration curve on the same run to afford the concentration of 6BIO.

2.6.1 Accuracy and precision

Precision and accuracy were evaluated by analyzing the samples at the three QC concentration levels, along with the LLOQ and ULOQ at five replicates during three validation days. The concentration of each sample was determined using the calibration curve prepared on the same day. Precision was expressed using the coefficient of variation (CV, %) between the replicate measurements. Accuracy was defined as the % relative error, .i.e. relative deviation in the determined concentration of a standard from that of its nominal concentration expressed as a percentage. The precision required was CV% within 15%, and accuracy were required not to exceed ±15%.

2.6.2 Specificity and selectivity

The interference of endogenous compounds in mice plasma was assessed as follows: Six blank plasma from six different mice were processed without addition of 6BIO and I.S, and were quantified using a valid calibration curve in order to test for possible interference at the retention time of 6BIO and the IS. For all the rat plasma batches, the peak area measured in the blank plasma sample had not to exceed 20% of the LLOQ peak area for 6BIO, and 5% for IS.

2.6.3 Calibration model, LLOQ and ULOQ

The calibration model curves for 6BIO was assessed by analyzing nine calibration standard samples in the range of 5.0 – 200 ng/mL in duplicate, employing the peak-area ratio of each analyte to that of the IS *versus* its plasma concentration, A quadratic weighted ($1/x$) least squares regression approximation has been selected as the most appropriate model.

The LLOQ was defined as the lowest concentration on the calibration curves of 6BIO measured with acceptable precision and accuracy (i.e. within ± 20% bias of the nominal value and with a %CV less than 20%) and a signal to noise ratio of at least 5. The ULOQ was defined as the highest concentration on the calibration curves of 6BIO.

2.6.4 Recovery (extraction efficiency)

Recovery of 6BIO after protein precipitation was estimated at the three QC concentration levels, as well as to the LLOQ and ULOQ by comparing the mean peak area ratios of the 6BIO to IS in pre- spiked extracted samples with post-spiked extracted blank samples, representing 100% recovery. In each case, five replicates were analyzed.

2.6.5 Matrix effect

The matrix effect was tested by comparing post-spiked blank samples to the corresponding standard saples at three QC concentration levels, as well as at the LLOQ and ULOQ. In each case, five replicates were analyzed. A value of 100% is indicative of no matrix effect, a value >100% suggests ionization enhancement and a value <100% suggests ionization suppression.

2.6.6 Carry over

For the evaluation of carryover effects, two plasma sample at the ULOQ level and two processed blank plasma were analyzed in consecutive LC-MS/MS runs. For acceptance, the peak areas of the blank sample should not exceed 20% of the peak areas obtained at the LLOQ, and 5% for the IS.

2.6.7 Stability

In order to simulate the experimental conditions that an unknown sample may be exposed during a routine analysis, free/thaw, autosampler, short term, and long term stabilities of samples were carried out and always compared to that of a freshly prepared sets of calibrations and QC samples. At each concentration level, the precision expressed as CV% had not to exceed 15%, and the accuracy expressed as %Er had to be within ±15% of the nominal value.

2.6.7.1 Three freeze - thaw cycles

Five replicates at the three QC concentration levels, LLOQ and ULOQ were subjected to three freeze/thaw cycles (from freeze temperature of -20 ºC to room temperature) and were quantified using the developed methodology.

2.6.7.2 Short term stability

Five replicates at the three QC concentration levels, LLOQ and ULOQ were stored for 6 h at room temperature before processing and analysis.

2.6.7.3 Autosampler stability

Five replicates at the three QC concentration levels, LLOQ and ULOQ were processed and stored at in the autosampler at 8 °C and protected from light, for 10 h and then analyzed.

2.6.7.4 Long term stability

Long-term stability of samples was evaluated by analyzing QC, LLOQ and ULOQ samples that were stored for 20 days at -80 °C.

2.6.7.5 Robustness

The robustness study was carried out to evaluate the influence of small deliberate variations of the optimized chromatographic conditions. Three different parameters were altered, within a 5-10% margin of the value used for the analysis, in order to evaluate the robustness of the proposed methodology. These parameters were the flow rate, the column temperature and the percentage of acetic acid in the aqueous phase. Thus, the values studied were 450, 500 and 550 μL/min for the flow rate, 22, 24 and 26°C for the column temperature and 0.095, 0.1 and 0.105% for the percentage of acetic acid in the aqueous phase. This study was performed at the LLOQ, ULOQ and the QCs levels (n = 5). The evaluation of the results was accomplished by ANOVA.

2.7 Pharmacokinetic study

The validated methodology was applied to determine the plasma concentration of 6BIO in mice (n=3) up to 24h. 6BIO solutionin aqueous Solutol (30% w/v) was administered to mice at a single dose of 50 mg/kg body weight (BW) by oral gavage. Blood samples were collected in microcentrifuge tubes containing EDTA anticoagulants at intervals of 0, 5, 15, 30 min, and 1, 4, 6, 8, 12, and 24 h . Then, blood samples were centrifuged at 1500 rpm for 5 min at room temperature. The obtained plasma samples were transferred into clean microcentrifuge tubes and frozen at -80^{o}C.

Mean plasma concentrations of 6BIO after oral administration versus time curve were generated in Microsoft Excel. The pharmacokinetic (PK) parameters were determined by non-compartmental analysis of the individual plasma concentration profiles using the PKsolver (add-in program for Microsoft Excel) The PK parameters determined were the maximum concentration (C_{max}), the time to reach the maximum concentration (T_{max}), the terminal elimination half-life ($t_{1/2}$), the elimination

rate constant (*Ke*), the area under the curve from time zero to the last detectable sampling point after administration ($AUC_{0\rightarrow last}$) and the area under the curve extrapolated to infinity ($AUC_{0-\infty}$),(calculated with the linear/log trapezoidal method), the mean residence time (MRT), the volume of distribution at terminal phase (Vz), and the clearance (CL).

3 Results and Discussion

3.1. Optimization of UHPLC-ESI MS/MS conditions

Despite the long history of indirubins in traditional, industrial and clinical practices, reported methods for their quantitation in biofluids are very scarce. The first analysis of indirubins in rat plasma was carried out using an HPLC-UV method (29). The limitations of this study were the use of a low resolution and non-specific detection technique (UV), as well as the lengthy chromatographic run times (env. 20 min). Hang et al. developed an HPLC-MS/MS method for the quantitation of mesoindigo – an indirubin isomer – and its reductive metabolites in rat plasma (30), with total analysis time reaching 30 min, which is a drawback in the analysis of a large number of samples. Therefore, the main aim of this study was to develop an analytical methodology featuring short analysis time for the quantitation of 6BIO in rat plasma. The UHPLC separation methodology was employed due to its inherent capability of affording ultra-high resolution chromatographic runs in a considerably short analysis time. The selected mobile phase (ACN and H_2O containing 0.1% FA) resulted in separation between 6BIO and the IS in 4.4-min run time (Figure 2). This ultra-fast chromatographic separation of the analytes was combined with MS/MS spectral detection, thus providing enhanced specificity and sensitivity. The MS/MS spectra from a Linear Ion Trap based quantitative bioanalysis assay allows not only the separation and differentiation of

endogenous compounds in biological matrices from the drug compound of interest or its metabolites, but also the elimination of any questions about false-positive data.

The first step of our method development was to acquire the high resolution Fourier transform full scan and MS/MS spectra of 6BIO in both positive and negative mode. For this purpose, a 10 μg/mL solution diluted in MeOH:H_2O (1:1, v/v) was directly infused into the MS system at a flow rate of 5 μL min^{-1}. The ESI interface parameters and the ESI probe position were also optimized for obtaining the maximum abundance of precursor and product ions. In the full scan mode, 6BIO exhibited two isotopic peaks: 353.9872 and 355.9852 (negative mode) vs 356.0027 and 357.0015 (positive mode). Both isotopes were taken into consideration to generated MS/MS spectra as summarized in table 1.

Linear Ion Trap full scan and MS/MS spectra of 6BIO were further acquired using the same conditions utilized for Orbitrap acquisition. For MS/MS experiments, the collision energy voltage for the effective fragmentation of the selected precursor ions of 6BIO was optimized over the range of 5-35% with a 2% step, and it was shown that 17% provided optimum fragmentation of 6BIO in negative mode and 19% in positive mode (Figure 2).

In both Orbitrap and Linear Ion Trap acquisitions, negative mode provided greater S/N ratio as compared to positive mode. However, the Linear Ion Trap rather than the Orbitrap provided more stable measurement since CV % values obtained employing Orbitrap analysis were in general > 35% and usually 10-fold higher than those in the case of Linear Ion Trap. Furthermore, the CV % and RE % remained the same whether selecting either isotope ion or both. Considering all the above, the Ion Trap operation at negative mode was selected for the analyses with a CID of 17% for the selected 354 ion of 6BIO. The IS MS/MS spectrum was acquired using a 35% CID as

previously reported (40). Product ions of 6BIO and IS after fragmentation with the Linear Ion trap are shown in figure 3.

The chromatographic conditions were optimized for the rapid and efficient separation of 6BIO and IS from plasma components. For this reason, a 5 cm length C18 silica column was chosen with acetonitrile as the organic mobile phase over methanol as this combination gave the best results in terms of peak shape (width, symmetry, sharpness) and accelerate the analysis time. Further optimization was achieved by adding 0.1% acetic acid in the aqueous mobile phase enhancing therefore the ionization efficiency in comparison to various percentages of formic acid. Under the optimized chromatographic conditions 6BIO was eluted at 3.08 min. and afromorsin (IS) at 2.52 min (Figure 4).

3.2. Sample preparation

Numerous methods were evaluated for the sample preparation procedure. Specifically, (i) protein precipitation with cold mixture of acetonitrile/methanol at various ratios (1/9, v/v), (3/7, v/v), (1/1, v/v), (7/3, v/v), and (9/1, v/v); (ii) liquid-liquid extraction, and (iii) SPE extraction. Amongst all, protein precipitation with cold acetonitrile/methanol (9:1, v/v) was chosen as it presented the highest recovery factor, the least matrix effect and was the less time consuming presenting also adequate sensitivity for the PK study of 6-BIO concentration after its administration to mice.

3.3. Choice of the IS

Afromorsin was selected as the IS mainly due to the fact that it is a readily available, inexpensive, not harmful and stable substance that could be ionized in the negative ionization mode. Moreover, afromorsin was eluted before 6BIO and was well separated from 6BIO without interference from

endogenous compounds in rat plasma, whereas its chromatographic characteristics were appropriate.

3.4. Method validation

3.4.1. Selectivity

The selectivity of our method refers to its ability to select and determine particular 6BIO and IS in plasma without interfering with the other metabolites. Therefore five plasma sample at the LLOQ were analyzed. The selectivity precision (CV %) was 9.62%, and the accuracy (RE %) was 1.14% (Table 2). Since the precision was below 20%, and the accuracy was within ±20% at the LLOQ, this analytical method for the quantification of 6BIO in rat plasma was shown to be selective.

3.4.2. Specificity

The specificity of the proposed analytical methodology was assessed by the analysis of six blank drug-free plasma samples. Analysis of these blank samples under the same conditions revealed no interference peaks from endogenous plasma components at the retention times of 6BIO and IS; the peak areas measured in the blank plasma sample were equal to 0.00% and, hence, less than 20.0% of the LLOQ average peak area, indicating that this analytical method is specific for 6BIO and suitable for bioanalysis. The representative chromatograms of blank rat plasma and plasma spiked with 6BIO at LLOQ and ULOQ and IS are shown in Figure 4.

3.4.3. Calibration range, calibration model, and LLOQ

Having no idea of the concentration of samples to be analyzed, and in order to avoid working with either a very large or a very narrow calibration curve, we firstly performed a preliminary calibration curve of 6BIO alone over the range 1 – 5000 ng/mL along with representative plasma

samples of the PK study. After analysis of the data, our concentration range was narrowed to 5 – 200 ng/mL; final calibration curves were constructed by plotting the peak area ratios of 6BIO to IS of plasma and calibration standards versus nominal concentrations of 6BIO. The calibration model was selected based on the analysis of the data by linear and non-linear regression as well as with and without weighting. The curve fitting was based on the simplest model that exhibited the highest correlation coefficient along with the lowest RE% on the back-calculated values. Calibration curves of five different lots of plasma calibration standards were constructed (Table 3).

The best fit and least square residuals for the calibration curves were achieved employing a quadratic model with 1/x weighting factor. The correlation coefficient (r^2) of the calibration curve was > 0.998, indicating good correlation. The back calculated values obtained are within the proposed 15% margin of the nominal concentrations indicating the adequacy of the proposed model. The LLOQ of 6BIO with the proposed method was determined to be 5 ng/mL fulfilling the specification that RE % and CV % should fall into the 20% margin and the S/N the analyte should be at least 5 (at least 5 times the signal of a blank sample).

In order to clarify if the calibration curves were the same an extra sum of squares F-test has been carried out. The results show that the calibration curves obtained do not differ statistically and therefore each one could be substituted either by one other in the set or could be described by one common curve. The consensus curve can be expressed by the following equation:

$$Y=0.00015(\pm0.00014)*X^2+0.97(\pm0.02)*X+0.20(\pm0.39)$$

The numbers in parentheses denote the standard error. The back calculated values calculated by the equation exhibit RE % values less than 15% for all points whereas the coefficient of the determination is better than 0.998.

3.4.4. Accuracy and precision

The intra- and inter-day precision and accuracy were evaluated at the three QC levels as well as at the LLOQ and ULOQ levels (Table 4). As shown in Table 4 the intra-day precision does not exceed 10.11 % whereas the inter-day was better than 9.62 % in every case. The RE %, indicating the accuracy of the method does not exceed 13.29%. Therefore, this bio-analytical method proved to be precise and accurate.

3.4.5. Recovery and matrix effect

In order to evaluate the recovery of 6BIO, twenty-five blank mouse plasma samples from five different animals were processed without addition of 6BIO and concomitantly they were spiked with 6BIO at the LLOQ, ULOQ, and QCs concentration levels (post spiked samples). The responses obtained represented the 100% recovery reference. The extraction recovery was calculated by dividing the peak area ratio of the post-spiked samples by the corresponding peak area ratio of the pre-spiked samples (spiked before protein precipitation). Similarly, the potential ion suppression or enhancement due to mouse plasma components (matrix effect) was evaluated by dividing the peak area ratio of the post-spiked plasma samples by the corresponding peak area ratio of working standard solutions diluted in MeOH:H2O (1/1, v/v). The extraction recovery of 6BIO was between 97.1 and 124.4 % across the entire range (5 - 200 ng/mL), while the matrix effect was between 73.8 and 85 % for 6BIO (Table 5).

3.4.6. Carry-over

The impact of the carry-over on blank mouse samples following the highest calibration sample was assessed for both 6BIO and IS. The average carry-over was 1 % (below 20%) for 6BIO and 0.04 % (below 5%) for the IS, indicating that the carry-over has no impact on the results.

3.4.7. Stability

No significant degradation have been observed under the stress condition imposed as described during the stability study (Table 6). Therefore, no special precautions are advised besides the ones described.

3.4.8. Robustness

Upon variation of the flow rate, the column temperature, and the percentage of acetic acid in aqueous phase, the retention time shift of 6BIO and IS remained statistically not significant at the 95% level using ANOVA.

3.5 Pharmacokinetic Study

This validated method was successively applied to a pharmacokinetic study of 6BIO in mice. The mean plasma concentration – time curve after a single oral administration of 6BIO at 50 mg/kg BW is shown in Figure 5.

The main pharmacokinetic parameters of 6BIO calculated using PKsolver software are summarized in Table 7. After administration of 6BIO, the mean maximum concentration (C_{max}) was 118.2 ± 58.7 ng/mL, and the area under the concentration-time curve ($AUC_{0\text{-}Inf}$) was 155.32 ± 34.99 ng*h/mL. The plasma concentration of 6BIO decreased rapidly and was eliminated from the plasma with a half-life time ($t_{1/2}$) of 0.72 ± 0.09 hours.

This study with an oral administration of 50 mg/kg BW validated the bioanalytical method developed and could be suitable to detect 6BIO levels in mouse plasma. Overall, low bioavailability and rapid decrease of 6BIO concentrations in plasma is observed. The low bioavailability could be attributed to the low solubility of 6BIO (sparingly soluble in aqueous

buffres, 0.3 mg/ml in a 1:2 solution of DMSO:PBS (pH 7.2)). The rapid decline of plasma concentrations might result from rapid distributions to other tissues. In future PK studies, we will investigate the distribution of 6BIO to other tissues after oral administration and the PK after intravenous injection can improve the plasma bioavailability of 6BIO.

4. Conclusion

The described UHPLC-ESI-MS/MS analytical methodology enables the rapid and selective determination of 6BIO in mouse plasma. The developed method is robust and presents high sensitivity, accuracy, precision, recovery, and stability. A small amount of plasma is required using this method (45 μL), making it applicable for many bio-analytical studies which generally present limited amount of sample. A time-saving one-step protein precipitation for sample pretreatment, combined to the rapid analysis time (4.4 min) render this method readily applicable in a further clinical study, comprising a large amount of samples and need for high-throughput method. The method was therefore applied to a preliminary pharmacokinetic study of 6BIO after oral administration of 50 mg/kg in mice. The pharmacokinetic profile of 6BIO was characterized for the first time and this showed a low bio-availability and a rapid elimination of 6BIO from the systemic circulation in mice.

Conflict of interest

Authors declared no conflict of interest.

Acknowledgements

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List of figures

527 Table 1: High resolution precursor and product ions of 6BIO in positive and negative ESI-Orbitrap

Precursor ions		CID	Product ions
Negative	353.9872	17	323.9893
	355.9852	17	325.9872
Positive	356.0027	20	326.0045
	357.0015	20	328.0035

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530 Table 2: Selectivity test at the LLOQ for 6BIO (n = 5)

Run	Replicate	Nominal level (ng/mL)	Calculated value (ng/mL)	Mean	SD	RE %	CV %
1	1	5	4.893	5.1	0.49	1.14	9.62
	2	5	4.491				
2	1	5	4.961				
	2	5	4.556				
3	1	5	5.882				
	2	5	5.873				
4	1	5	4.794				
	2	5	4.780				
5	1	5	5.210				
	2	5	5.130				

SD: standard deviation

RE %: deviation of mean from the nominal value

CV %: coefficient of variation

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Table 3: Calibrators and calibration curve parameters

Run	Nominal values (ng/mL)									r^2
	5	**10**	**25**	**50**	**75**	**100**	**125**	**150**	**200**	
1	4.9	9.2	24.4	51.0	71.7	105.3	122.9	151.2	207.7	0.998
	4.5	9.8	24.4	52.2	70.6	104.2	124.0	146.0	212.6	
2	4.6	9.4	22.6	49.7	78.5	101.6	125.0	150.2	220.7	0.999
	4.9	10.6	23.0	48.5	77.2	98.0	129.1	147.8	202.7	
3	4.3	12.1	25.2	49.9	84.5	99.6	116.9	137.1	206.6	0.992
	4.3	10.0	24.1	53.6	84.4	97.7	115.0	135.6	219.4	
4	4.7	9.9	26.3	52.1	80.6	99.1	118.7	129.9	204.8	0.998
	6.1	10.5	25.0	48.8	76.0	95.3	123.3	123.1	200.3	
5	5.4	10.4	23.7	52.3	84.7	92.3	120.4	146.5	201.8	0.997
	5.1	9.7	25.4	51.9	80.7	93.8	119.1	148.2	209.0	
Mean	**4.9**	**10.1**	**24.4**	**51.0**	**78.9**	**98.7**	**121.4**	**141.6**	**208.6**	
SD	0.56	0.83	1.13	1.68	5.09	4.25	4.21	9.60	7.07	
RE %	-2.32	1.44	-2.39	1.98	5.19	-1.32	-2.85	-5.62	4.28	
CV %	11.47	8.14	4.61	3.29	6.45	4.31	3.46	6.78	3.39	

Run 1: Y1 = 0.0025 + 0.011*X - 1.33e-005*X^2 r^2 = 0.998

Run 2: Y2 = -0.0052 + 0.013*X - 8.22e-006*X^2 r^2 = 0.999

Run 3: Y3 = 0.0002 + 0.008*X - 2.04e-006*X^2 r^2 = 0.992

Run 4: Y4 = 0.0123 + 0.007*X - 1.00e-006*X^2 r^2 = 0.998

Run 5: Y5 = 0.0002 + 0.007*X + 2.68e-007*X^2 r^2 = 0.997

541 Table 4: Within- and between-series precision (CV %), and accuracy (expressed as RE %) of

542 LLOQ, ULOQ and QC samples

Level	Found	Day1	Day 2	Day 3			Overall
		n =5	n = 5	Run 1 (n = 5)	Run 2 (n = 5)	Run 3 (n = 5)	n = 25
LLOQ (5 ng/mL)	Mean	**5.1**	**5.1**	**5.0**	**4.9**	**5.0**	**5.1**
	SD	0.49	0.40	0.35	0.14	0.21	0.06
	RE %	1.14	2.51	0.77	2.00	1.00	1.39
	CV %	9.62	7.79	7.02	2.89	4.29	1.14
LQC (7.5 ng/mL)	Mean	**7.6**	**7.8**	**7.4**	**6.5**	**8.4**	**7.3**
	SD	0.59	0.29	0.74	0.47	0.85	0.57
	RE %	1.83	4.10	1.93	13.29	12.06	2.33
	CV %	7.70	3.69	10.11	7.24	10.08	7.83
MQC (40 ng/mL)	Mean	**43.1**	**39.1**	**44.2**	**44.5**	**42.6**	**42.7**
	SD	1.89	1.37	1.44	1.50	2.42	2.16
	RE %	7.83	2.23	10.47	11.31	6.46	6.75
	CV %	4.39	3.51	3.27	3.37	5.69	5.05
HQC (175 ng/mL)	Mean	**185.3**	**175.1**	**185.2**	**186.5**	**181.8**	**183.0**
	SD	5.77	8.67	12.94	12.74	5.32	5.32
	RE %	5.86	0.05	5.85	6.54	3.86	4.59
	CV %	3.12	4.95	6.99	6.83	2.92	2.90
ULOQ (200 ng/mL)	Mean	**200.7**	**199.8**	**194.9**	**206.9**	**195.0**	**200.3**
	SD	15.44	9.53	0.74	7.85	2.83	0.64
	RE %	0.35	0.09	2.6	3.43	2.50	0.13
	CV %	7.69	4.77	0.38	3.79	1.45	0.32

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545 Table 5: Recovery of 6BIO in mouse plasma and matrix effect (n = 5)

Level	ng/mL	Matrix effect (%)	SD	RE %	CV %	Recovery (%)	SD	RE %	CV %
LLOQ	**5**	**73.8**	9.36	26.25	12.69	**98.5**	3.42	1.54	3.48
LQC	**7.5**	**83.6**	13.28	16.45	15.89	**109.0**	12.66	9.02	11.6
MQC	**40**	**85.0**	6.53	15.00	7.68	**97.1**	4.80	2.86	4.94
HQC	**175**	**82.8**	3.57	17.25	4.31	**94.6**	7.65	5.41	8.08
ULOQ	**200**	**79.5**	2.57	20.50	3.23	**124.4**	2.83	24.36	2.28

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548 Table 6: Stability results of 6BIO in mice plasma under various conditions (n = 5)

Level	Spiked Conc. (ng/mL)	Freeze-thaw (-20 ºC to 25 ºC)			Short-term (6h, 25 ºC)			Autosampler (10h, 8 ºC)			Long term (15 days, -80 ºC)		
		Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)
LLOQ	5	5.1	10.00	9.80	4.95	5.00	6.06	5.12	12.00	5.86	5.03	3.00	9.94
LQC	7.5	7.7	2.67	9.09	6.9	8.00	10.14	7.40	1.33	13.51	7.55	0.67	10.60
MQC	40	42.5	6.25	5.53	44.0	10.0	8.07	43.5	8.75	10.46	40.50	1.25	14.57
HQC	175	180.5	3.14	5.82	173.5	0.86	3.07	173.5	0.86	4.32	170.5	2.57	5.87
ULOQ	200	201.1	0.55	3.73	205.5	2.75	3.16	199.5	0.25	3.76	195.0	2.50	3.85

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551 Table 7: Non-compartmental pharmacokinetic parameters of 6BIO in plasma after a single oral
552 dose of 50 mg/kg BW to mice (n = 3).

Parameter	**Unit**	**Mean values**	**SD**
T_{max}	h	0.50	0.0
C_{max}	ng/mL	118.2	58.7
Elimination rate constant (Ke)	1/h	0.97	0.12
Terminal elimination half-life (t1/2)	h	0.72	0.09
AUC 0-t	ng/mL*h	155.3	35.0
AUC 0-inf	ng/mL*h	157.6	36.2
MRT 0-inf	h	0.98	0.16
Vz/F	(mg/kg)/(ng/mL)	0.35	0.13
Cl/F	(mg/kg)/(ng/mL)/h	0.33	0.09

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